APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1996, p. 4580–4586 Vol. 62, No. 12
0099-2240/96/$04.00+0

Enumeration and Biomass Estimation of Bacteria in Aquifer Microcosm Studies by Flow Cytometry

PAUL C. DELEO† AND PHILIPPE BAVEYE*

Laboratory of Environmental Geophysics, Cornell University, Ithaca, New York 14853

Received 24 May 1996/Accepted 1 October 1996

Flow cytometry was used to enumerate and characterize bacteria from a sand column microcosm simulating aquifer conditions. Pure cultures of a species of *Bacillus* isolated from subsurface sediments or *Bacillus megaterium* were first evaluated to identify these organisms' characteristic histograms. Counting was then carried out with samples from the aquifer microcosms. Enumeration by flow cytometry was compared with more-traditional acridine orange direct counting. These two techniques gave statistically similar results. However, counting by flow cytometry, in this case, surveyed a sample size 700 times greater than did acridine orange direct counting (25 μl versus 0.034 μl) and required 1/10 the time (2 h versus 20 h). Flow cytometry was able to distinguish the same species of bacteria grown under different nutrient conditions, and it could distinguish changes in cell growth patterns, specifically single cell growth versus chained cell growth in different regions of an aquifer microcosm. A biomass estimate was calculated by calibrating the total fluorescence of a sample from a pure culture with the dry weight of a freeze-dried volume from the original pure culture. Growth conditions significantly affected histograms and biomass estimates, so the calibration was carried out with cells grown under conditions similar to those in the aquifer microcosm. Costs associated with using flow cytometry were minimal compared with the amount of time saved in counting cells and estimating biomass.

Enumeration of microorganisms and characterization of their physical features (size and shape, etc.) are routine practices in environmental microbiology. The progenitors of some of today's enumeration techniques go back to the days when Robert Koch incorporated agar-agar into plating media (26). A number of enumeration protocols still popular at present were developed decades ago, such as direct counting (3, 7) and the most-probable-number technique (10, 23). Although these techniques are routinely used, they have a number of limitations (8) and are often extremely labor-intensive. Even the comparatively simple most-probable-number method requires extensive preparation (10). Bright-field or phase-contrast direct counting methods are also in common usage but have the disadvantages of not distinguishing between bacteria and similarly shaped particles or between viable and nonviable organisms (17). The use of fluorescent dyes with various specificities in epifluorescence microbiology proved to be a vast improvement for enumeration (12). Nucleic acid-specific dyes provide differentiation of cells from other nonbiological materials, and dyes exist which permit one to distinguish between viable and nonviable cells (24). Very specific immunofluorescent dyes allow the identification of target organisms (37, 43). In spite of these recent improvements, epifluorescence direct count techniques still require a sizeable effort to obtain satisfactory results.

Automated techniques for enumeration and determination of biomass and biovolumes existed for a number of decades although until recently they were not widely used. These include electrical resistance techniques, such as the Coulter counter and similar devices (28), and photoelectric techniques, such as flow cytometry. The Coulter counter was developed in the mid-1950s (11) and was adopted quickly. Photoelectric counting techniques were first proposed in the 1930s (35), but working devices were not available until the late 1940s (16, 20, 21). Technological developments of particle counters and flow cytometers continued through the 1960s, with particle counters gaining wide acceptance. The advent of the microprocessor, however, led to great progress in flow cytometry. The added power of fluorescence techniques established flow cytometry as a primary tool in biological sciences over the past 15 years (for a more complete discussion of the history of flow cytometry, see references 34 and 39).

Flow cytometry is a tool with great potential for use in environmental microbiology because of the quantity and quality of data it provides in a timely fashion. Flow cytometry typically provides data relative to five parameters: forward angle (~10 to 15°) and side angle (90°) scatter of the incident illumination source and three fluorescence channels (blue, green, and red). This technology benefits from the many advantages of fluorescence techniques in such areas as identification with specific dyes or probes, physiological investigations, and study of cells with autofluorescent components. One may also use forward scatter and side scatter data to differentiate among numerous populations or observe changes in populations. Flow cytometry has the added advantage of operating with large samples, up to approximately 200 μl. Several investigators discuss concentration methods to decrease detection limits for cases in which target organisms are in low number (32, 49). By contrast, sample volumes associated with epifluorescence techniques are typically several orders of magnitude smaller. Sample sizes for epifluorescence techniques can be increased through automation of microscopy (6), counting more fields in acridine orange direct counting (AODC) (22), or filtration of samples with epifluorescence counting (30), but the effort associated with decreasing the detection limit may be prohibitive.

Flow cytometry's primary use to date was in eukaryotic cell

* Corresponding author. Mailing address: Laboratory of Environmental Geophysics, Bradfield Hall, Cornell University, Ithaca, NY 14853. Electronic mail address: pcb2@cornell.edu.

† Present address: Malcolm Pirnie, Inc., White Plains, NY 10602.

biology. It was not routinely used in microbiology because, until recently, the electronics were not sensitive enough to detect bacteria which are orders of magnitude smaller than eukaryotes (33). Flow cytometry in microbiology initially dealt with analysis of the cell cycle of *Escherichia coli* (44, 45). More recently, the technique is used in environmental microbiology to characterize microbial populations obtained from aquatic environments such as lake water (38), seawater (29), and sewage (50, 51). Likewise, the technique is utilized to investigate eukaryotic microorganisms of environmental significance, such as phytoplankton (27), *Giardia* spp. (47), and *Cryptosporidium* spp. (48). Few reports exist describing the use of flow cytometry with organisms associated with soils or other solid matrices such as compost (9, 14, 36, 53), and there are at present no reports of work with microorganisms from subsurface sediments or aquifer material.

In this context, the objective of this study was to develop a reliable technique based on flow cytometry for rapid enumeration and biomass determination of bacteria used in aquifer microcosm studies. AODC was adopted as the standard for comparison. Epifluorescence direct counting is a commonly used technique for measuring total numbers of microorganisms (25) and is widely used for enumeration of organisms from subsurface soils (15), subsurface sediments (4, 41), and aquifers (31, 52). Previous research has shown that flow cytometry provides results comparable to epifluorescence direct counts for fluorescent beads (30) and that biomass estimations could be made by correlating nucleic acid stain fluorescence and DNA content (5). The method described in this study yielded bacterial counts that were statistically similar to AODC, in a fraction of the time. In addition, it provided a biomass estimate. This new counting method required modification of several standard procedures for extraction and preparation of soil microorganisms for microscopic examination, as well as the optimization of flow cytometric parameters for the organisms of interest.

MATERIALS AND METHODS

Organisms. The development of a new flow cytometric counting method was a component in a study of the effects of predator-prey interactions on bacterial clogging in a laboratory aquifers microcosm. A *Bacillus* sp. (B0069) obtained from the Subsurface Microbial Culture Collection at Florida State University and *Bacillus megaterium* (19213) obtained from the American Type Cultural Collection (Rockville, Md.) were chosen because they provide the most favorable yield of an amebal predator when grown with this predator in batch culture. *Bacillus* sp. (B0069) was isolated from Tobacco Road formations (34 m) from borehole P24 at a Savannah River site in Aiken, S.C. Characterization was based on 16S rRNA sequencing by subsurface Microbial Culture Collection. Additional physiological tests showed the following results: catalase, positive; Voges-Proskauer test, negative; growth at 65°C, negative; hydrolysis of starch, positive; acid and gas from glucose, negative; width of >1.0 μm, negative; pH of <6.0 in Voges-Proskauer broth, positive; and growth under anaerobic conditions (GasPak), negative. These physiological characteristics are similar to those of *Bacillus circulans* (42).

Media. Bacterial pure cultures were maintained in PTYG broth (1) ([amounts expressed in grams liter^{-1}] peptone [Difco], 5.0; Tryptone [Difco], 5.0; yeast extract [Difco], 10.0; D-glucose, 10.0; $MgSO_4 \cdot 7H_2O$, 0.6; $CaCl_2 \cdot 2H_2O$, 0.07). A defined minimal medium with a simple sugar as carbon source was used in the aquifer microcosm studies. *Pseudomonas* basal mineral medium (P-broth) was found to be suitable for growth of the *Bacillus* sp. (1). P-broth was prepared by adding the following elements to 900 ml of deionized water: (amounts expressed in grams) K_2HPO_4, 6.525; KH_2PO_4, 8.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.1; 5.0 ml of trace element solution consisting of (amounts in grams liter^{-1}) H_3BO_4, 0.232; $ZnSO_4 \cdot 7H_2O$, 0.174; $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$, 0.116; $CoSO_4 \cdot 7H_2O$, 0.096; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.022; $CuSO_4 \cdot 5H_2O$, 0.008; and $MnSO_4 \cdot 4H_2O$, 0.008. The solution was then sterilized by autoclaving at 121°C and 15 lb/in^2 for 15 min, and 100 ml of the solution containing 9.0 g of D-glucose was later filter sterilized into the bulk salts medium. Bacteria were grown in P-broth for inoculation into the aquifer microcosms.

Experimental apparatus. Aquifer microcosms were 15-cm-diameter Lucite cylinders (10 cm in height), capped at the ends by 25-mm Lucite plates that were machined to fit the faced ends of the cylinder. Rubber gaskets were used to prevent leakage. Plainfield (Cass County, Ill.) sand sieved to a uniform 63- to 150-μm fraction was treated with 1 M sodium acetate at pH 5 to remove carbonates, with 6% H_2O_2 to remove organic matter, and with sodium hydrosulfite to remove iron compounds as described by Vandevivere and Baveye (46) in order to eliminate possible chemically mediated mechanisms of clogging and to control carbon content in the columns. The sand was autoclaved at 130°C and 26 lb/in^2 for an hour and then added to the columns. Sand was held in place by a stainless steel mesh. Assembled columns were sterilized by overnight exposure to ethylene oxide gas. Afterwards, columns were saturated with sterile 1% P-broth and inoculated with the *Bacillus* sp. or *B. megaterium*, which were harvested from cultures grown in P-broth. After a 24-h acclimation period, 1% P-broth was allowed to flow through the columns at a rate of 1 pore volume per h for 30 days, at which time they were disassembled and the samples were removed.

Extraction of microorganisms. The procedure for extraction of organisms from column samples was adapted from a commonly used method described by Ghiorse and Balkwill (19). It utilizes a chemical dispersant and shaking to remove organisms from sand particles. Lindahl and Bakken (32) examined physical and chemical extraction procedures in detail and found the chemical extraction procedures inferior for field soils because aggregation, clays, and organic matter prohibited dispersion of the microorganisms. However, the porous medium used in the present study was structureless and devoid of clay or organic matter, so the use of a chemical extraction procedure seemed appropriate.

Glassware, pipets and pipet tips were rinsed twice in 70% ethanol prior to autoclaving at 121°C and 15 lb/in^2 for 15 min. Solutions were passed through a 0.2-μm-pore-size filter and autoclaved to prevent microbial contamination.

Samples were taken from a microcosm by using a 15-cm length of 29-mm (outside diameter) steel pipe with a 1-mm wall having one end ground to make a sharp edge. The coring device was rinsed with 70% ethanol and allowed to dry prior to sampling. A core was taken from the entire length of the microcosm and divided into 10 equal parts. Extraction was accomplished by placing three 2.5-g subsamples of sand from each of the 10 equal parts into three tared, stoppered, 250-ml Erlenmeyer flasks. To the flasks was added 22.5 ml of 0.1% sodium P_i ($Na_4P_2O_7 \cdot 10H_2O$) solution adjusted to pH 7.0 with HCl in order to disperse cells from the sand particles. Flasks were placed on a rotary shaker table at 150 to 160 rpm at 25°C for 60 min.

Flow cytometry. Samples were prepared for flow cytometry by removing 1.0 ml of the extraction solution, which was allowed to settle for 1 min, and transferring it to a sterile 20-ml vial containing 8.765 ml of 0.1% sodium P_i solution. To fix cells in the sample, 0.135 ml of 37% formaldehyde (not sterilized or filtered; Sigma, St. Louis, Mo.) was added to the viral. After 20 min, 0.1 ml of a filtered 1-mg/ml solution of propidium iodide (Sigma) was injected into the vial to stain the cells. After 20 min, flow cytometry was performed with a Coulter Electronics (Hialeah, Fla.) profile analyzer at the Flow Cytometry and Imaging Facility at Cornell University. Propidium iodide fluorescence was excited by using all lines from an air-cooled argon-ion laser (principally a 488-nm wavelength, with a smaller contribution from 458 and 514 nm), and the emission detected through a series of filters was monitored. The first filter was a 488-nm dichroic filter which reflects scattered laser light to monitor 90° light scatter. A 457- to 502-nm laser-blocking filter and an A_{515} filter served to block the remaining scattered laser light and pass all the fluorescence to a 550-nm short-pass dichroic filter which transmitted the green emission to a 525-nm band-pass filter and 530-nm short-pass filter before reaching the detector (FL1). Longer-wavelength-reflected emission then passed through a A_{590} filter before reaching the detector (FL2).

Contour plots were generated by analyzing the 64 × 64 data matrix of FL2 versus side scatter or forward scatter versus side scatter with DeltaGraph version 3.5 (DeltaPoint, Monterey, Calif.). Bacterial counts were measured in a predetermined bitmap. The bitmap was established by analyzing pure cultures of bacteria grown in P-broth prepared as described above. The number of bacteria counted in the predetermined bitmap was measured. A single parameter histogram was generated by bitmap counts against the FL2 channel number and was analyzed for multiple peaks. If multiple peaks were found, the channel values for the two peaks were compared to determine the number of cells associated with each peak. The peak with the lowest value was confirmed by microscopy to be associated with single cells.

Calibration for biomass was obtained by comparing the total fluorescence of a pure culture of bacteria with the weight of a freeze-dried sample from the same culture. The *Bacillus* sp. was inoculated into a flask containing 50 ml of 1% P-broth and placed on a rotary shaker at 160 rpm and 27°C. After 48 h, three 1-ml samples were taken from the flask and prepared for flow cytometry. The number of counts along the 256 FL2 channels was measured. Total fluorescence was calculated by multiplying the number of counts per channel by the channel strength and summing the results for all channels. The channel strength for FL2 was over a two-order-of-magnitude range and may be computed with the formula $1.024 \times 1{,}000^{(n/256)}$, where n, the channel number, is between 0 and 255. The corresponding biomass (dry weight) was determined by placing three 10-ml samples from the original culture into separate preweighed, dried centrifuge tubes and by centrifuging them at 10,000 × g for 10 min. The supernatant was pipetted off, and the tubes were placed in a freeze-drier (Refrigeration for Science, Inc., Island Park, N.Y.) at <10 μm Hg (1 mm Hg = 133.322 Pa) and −40°C for 24 hours. The tubes were reweighed, and biomass (dry weight) was

determined as the difference. Dry mass weight was then correlated with total fluorescence.

AODC. Slides were prepared for AODC by removing 1.0 ml of extraction solution which had been allowed to settle for 1 min and transferring it to a 20-ml vial containing 7.865 ml of 0.1% sodium P_i solution. To this was added 0.135 ml of 37% formaldehyde. Then, 1.0 ml of molten 1% aqueous Noble agar was added to the vial and mixed thoroughly. Two 5-μl samples from each vial were spread uniformly onto slides to cover the 1.0-cm^2 area of two 1.1-cm-diameter circles drawn on the bottom of the slides with a marking pen. The slides were placed in a covered area to allow uniform air drying. Smears were stained by adding a few drops of an aqueous solution of 0.01% acridine orange (Sigma) for 2 min, washed with 20 ml of 1 M NaCl, rinsed briefly with deionized H_2O, and allowed to air dry. Once dry, a couple of drops of filtered (0.2-μm-pore size) DABCO solution (0.1635 g of 1,4-diazabicyclo-[2,2,2]-octane, 5 ml of H_2O [Sigma]) were placed on each smear, and a coverslip was mounted and sealed with VasPar (50% petroleum, 50% paraffin). Slides were viewed under epi-illumination with a phase-contrast microscope (Carl Zeiss, Inc., Thornwood, N.Y.) with ×100 oil objective lens and a ×10 wide-field eyepiece. Cells were counted in 15 fields per smear.

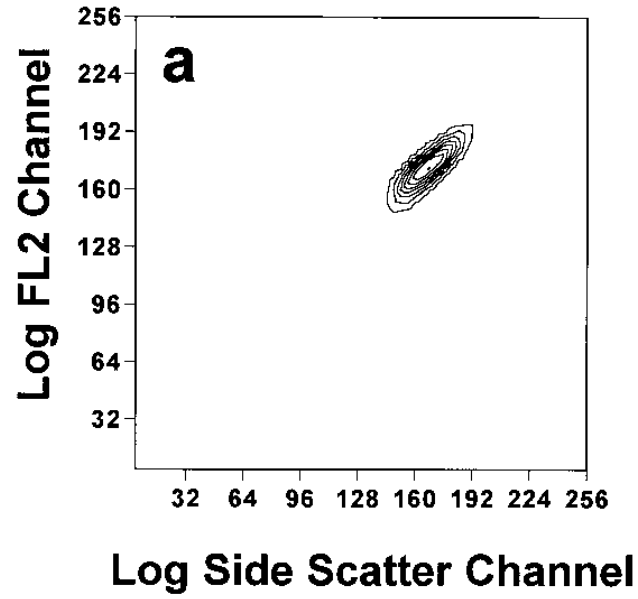
Statistical analysis. Sample variances of AODC and flow cytometry were tested by comparing the quotient of the square of the variances to an F-distribution for n_1-1 and n_2-1 degrees of freedom at $\alpha = 0.1$. A two sample independent t test was performed between the means of bacterial counts from AODC and flow cytometry as a function of depth in the column by using MINITAB Statistical Software (Minitab, Inc., State College, Pa.) release 8 for the Macintosh. Sample variances were pooled when appropriate. A t statistic was generated and compared with a t distribution for $t(n_1+n_2-2)$. The sample size for AODC (n_1) was 6, and the sample size for flow cytometry (n_2) was 3.

RESULTS AND DISCUSSION

The organisms used in our microcosm studies were first examined in pure culture. Populations of the *Bacillus* sp. gave symmetric two-parameter FL2 versus side scatter histograms when grown on a rich PTYG medium, as seen in the contour plot in Fig. 1a. Cells grown in a defined inorganic medium with glucose (P-broth) still had a distinct FL2-versus-SS histogram; however, it was not as symmetric as the first and noise was more noticeable (Fig. 1b). The two bacterial species used in this study are relatively large, so detection was not difficult. Still, there are numerous reports of utilization of flow cytometry with samples containing smaller gram-negative bacteria (51) or starved cells (9). One would anticipate that with the constant improvement in the sensitivity of the detection systems, the use of flow cytometry for analysis of even the smallest bacteria found in nature will soon be practical.

A change in one growth characteristic of *B. megaterium* was detected by flow cytometry. Organisms in samples from the first 40 mm of the column near the flow inlet were in a population of single cells (Fig. 2a). Organisms in samples from the remaining 60 mm of the column were in two populations, one of single cells and one of multiple cells (Fig. 2b). Observations using epifluorescence microscopy confirmed a large number of cells growing in chains in the latter samples, which would be consistent with the multiple cells observed in Fig. 2b. It was determined that the population of multiple cells on average had 10 times the fluorescence of the population of single cells. It was inferred that the average length of the cell chains was ten cells. This factor was applied to cells in that range for the calculation of counts.

Flow cytometry demonstrated utility in identifying multiple species of prokaryotes and eukaryotes in samples (51). Likewise, we were able to distinguish between our bacteria and an amebal predator (13a), but accurate detection of both was difficult when the difference in their numbers was great, as was often the case in our aquifer microcosms. Bacteria were typically found to be 3 to 5 orders of magnitude higher in number than protozoa (13). The difference between the numbers of organisms should be 2 orders of magnitude or less for optimal identification. For samples in which the numbers of organisms differ by several orders of magnitude, it is best to run the samples multiple times under optimal settings for each species.



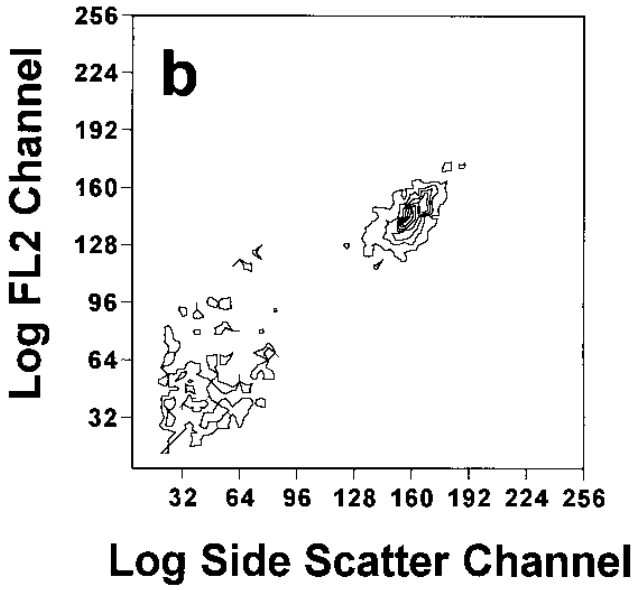


FIG. 1. Contour plot of counts of *Bacillus* sp. based on FL2 channel versus side scatter channel. (a) Growth on PTYG. Contours are expressed in increments of 100, starting at 100. (b) Growth on P-broth. Contours are expressed in increments of 5, beginning at 5.

A key objective of this research was to demonstrate that flow cytometry yields bacterial numbers that are comparable to those obtained with counting procedures commonly used in environmental microbiology. The variances of AODC and flow cytometry counts were found to be statistically similar at $\alpha = 0.1$ for all the samples, except at 5 mm where the variance associated with flow cytometry numbers was statistically lower than that obtained by AODC (Fig. 3). Sample variances were

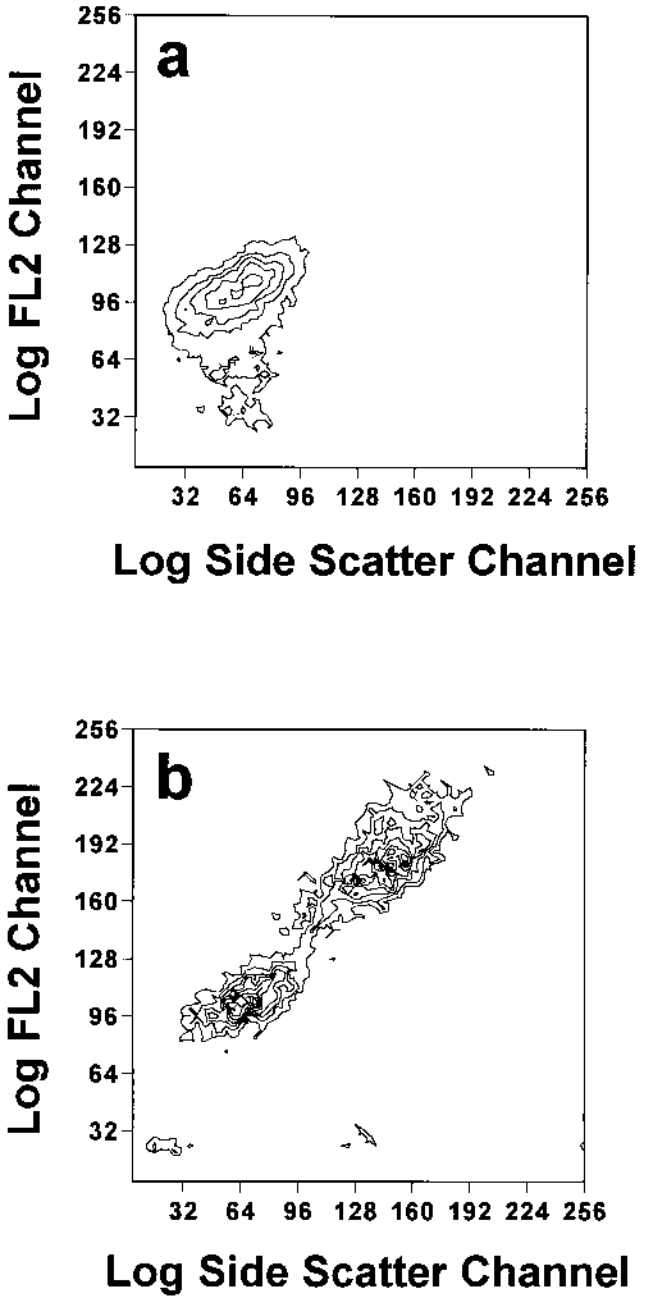


FIG. 2. Contour plot of counts of *B. megaterium* from an aquifer microcosm based on FL2 channel versus side scatter channel. (a) Sample taken from 5 mm beneath the nutrient source inlet. Contours are expressed in increments of 25, beginning at 25. (b) Sample taken from 45 mm beneath the nutrient source inlet. Contours are expressed in increments of 5, beginning at 10.

pooled for all samples except 5 mm for a *t* test. The mean counts by AODC and flow cytometry were found to be statistically similar at the 95% confidence interval for all 10 samples.

Utilization of flow cytometry for enumeration of bacteria has a distinct advantage over AODC and other techniques. By flow cytometry 50,000 cells in a sample can quickly and easily be counted. In this case, samples were 25 μl and counting of each required 2.5 min. The volume surveyed by flow cytometry was 700 times greater than that by AODC (25 μl versus 0.034 μl). The time required to count a typical 30-sample set from a microcosm by flow cytometry was 1/10 that of AODC (2 h versus 20 h). One report of automated epifluorescence counting with a confocal laser scanning microscope and image analysis showed improved counting, with additional data on cell volume and the frequency of cell division (6). Still, the speed of counting for confocal laser scanning microscopy with image analysis was very slow compared with that of flow cytometry, as these investigators reported that 1,500 cells were counted in 10 fields of a single smear in 30 min. Similar data on cell volume and frequency of division can be obtained by both techniques. Also, the capital cost of a confocal laser scanning microscopy system with image analysis is similar to that for a flow cytometer, but the cost of using the Flow Cytometry Facility at Cornell University was $45/h. The cost of using the facility for analysis of a set of samples was minimal relative to the amount of time saved in counting and performing a biomass estimation.

Flow cytometry also has a distinct advantage over AODC for the detection limit one may achieve. It is possible to reach a detection limit of 6.1×10^3 cells per g (dry weight) if a conservative minimum cell count of 100 is observed for a 200-μl sample (the limit for the EPICS Profile) of a 10-fold dilution of a sand sample (22% water content). On the other hand, the detection limit for the AODC method is about 5×10^5 cells per g (dry weight) (18). A detection limit of 10^3 bacteria per g was reported for AODC by automated counting with a confocal laser scanning microscope (18), but this required counting more than 10,000 fields and took several weeks (18a).

Another objective of the research reported in the present article was to estimate the bacterial biomass in an aquifer microcosm. In this case biomass was correlated to total fluorescence intensity. Fluorescence is a function of the amount of fluorochrome in each cell. This is directly correlated with the nucleic acid content in the cell when a nucleic acid stain such as propidium iodide is used (24). Therefore, total fluorescence is indirectly correlated with the quantity of nucleic acids, although an actual measurement of nucleic acids was not made. Instead, we correlated total fluorescence with the dry weight of a sample of cells from a pure culture grown under conditions similar to those in the experimental apparatus. This was important because the mean fluorescence (and thus total fluorescence) of a population varied on the basis of the nutrient solution used, which was not surprising. The high fluorescence of cells grown on PTYG (Fig. 4) was consistent with multiple genomes per cell. As this medium contains all necessary amino acids and carbon for growth, one would expect to find a high nucleic acid content associated with multiple copies of DNA and RNA, as a result of DNA synthesis. The cells grown in defined P-broth were also highly fluorescent. Here we expected to find high nucleic acid contents rather than multiple genomes, since, on one hand, the carbon concentration is high and, on the other, cells must synthesize all their own amino acids and proteins. Cells taken from the experimental column had lower mean fluorescence as a result of a low nutrient concentration and minimal DNA and RNA content.

Many of the histograms had an artifact that occurred occasionally at the lowest channels of FL2 (Fig. 4). This phenomenon is not unusual and is attributed to young cells in a culture (9), interfering background fluorescence (36), and detection of unbound fluorescent probes (40). In this study, the artifact was found in blank samples and was magnified when the sample flow rate was increased. We concluded that residual matter on the inside of the flow lines was disrupted by the higher flow rates and organisms in the sample. This artifact is easily identified and did not affect results. Signals generated by blanks were removed from test samples.

The total fluorescence of an FL2 histogram of a pure culture

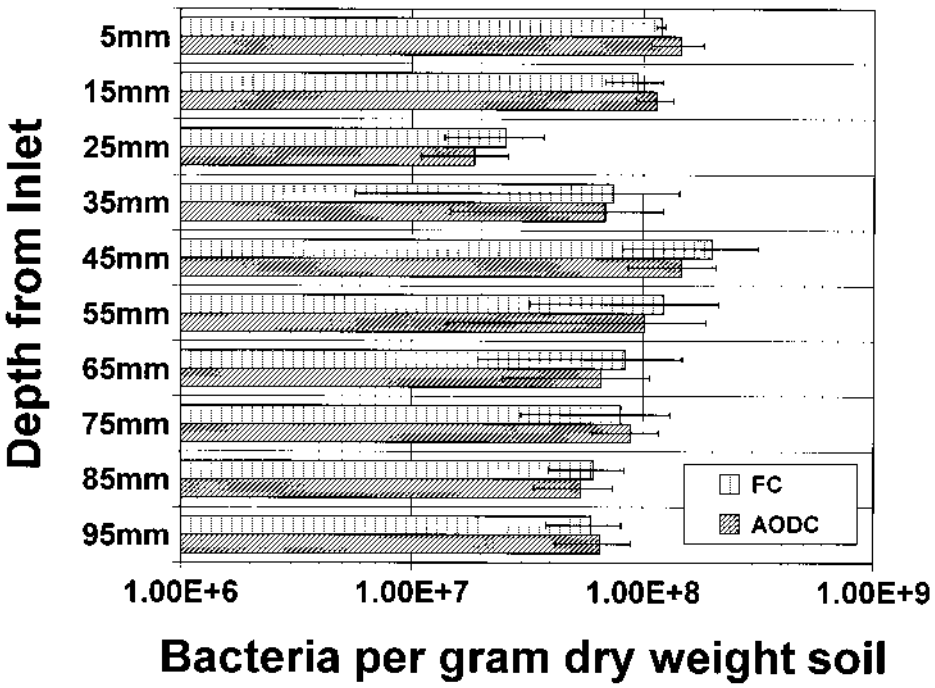


FIG. 3. Comparison of counts of *B. megaterium* from an aquifer microcosm based on flow cytometry and AODC.

grown in a 1% solution of P-broth was correlated to the weight of a 10-ml freeze-dried sample. This growth condition was nearly identical to that found in the experimental apparatus. The calibration yielded a result of 2.09 ± 0.94 mg (dry weight) of cells per 100,000 fluorescence units for the *Bacillus* sp. These coefficients are specific to the flow cytometry used and to the setting of the electronics and may not apply under different conditions.

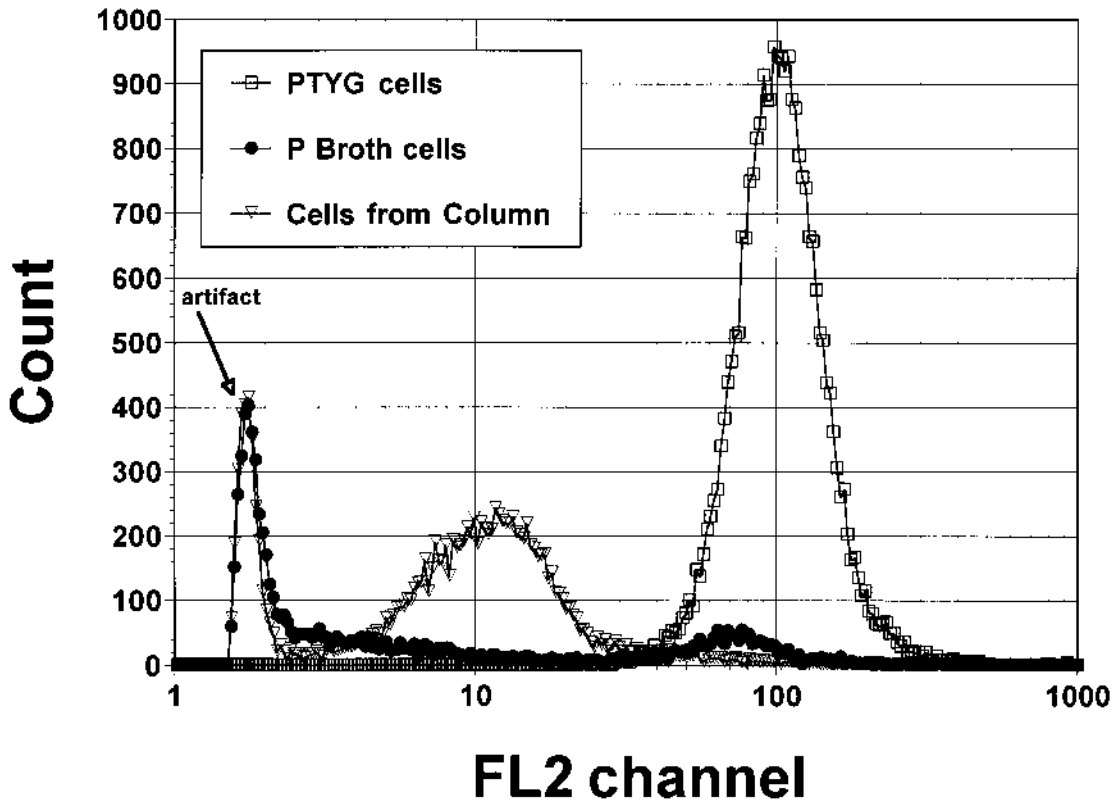


FIG. 4. Comparison of FL2 channel strength histograms for cells of *Bacillus* sp. grown under different conditions.

The measurement technique described in the present article was successful for enumerating bacteria and estimating biomass in an aquifer microcosm system filled with sand of narrow granulometry and uniform physical and chemical characteristics. Conditions similar to those are found in some aquifer materials; however, they are by no means the rule. Microbial enumeration of environmental samples by flow cytometry with more heterogenous compositions is at this stage still hampered by a number of technical problems such as aggregates in the sample solution that might clog the flow cytometer orifice, dispersion of organisms from aggregates and particles, and interferences that might mask organisms. These problems were investigated to some extent in other contexts. Coarse filtration of a compost extract was used to eliminate large particles while not significantly affecting the cell counts (14). Also, density gradient centrifugation was used to eliminate soil particles from bacterial suspensions (9). Problems with dispersion are a factor in all enumeration techniques, and there are a variety of physical and chemical means of dealing with them (2).

In summary, flow cytometry appears to hold much promise as a rapid and accurate method for the determination of bacterial cell numbers and biomass densities in aquifer materials. Results available for homogenous aquifer materials show that flow cytometry is as accurate as the AODC method and requires significantly less preparation time. Improvements in the counting method described in the present article are, however, still needed to deal adequately with very heterogenous and aggregated porous matrices.

REFERENCES

1. **Atlas, R. M.** 1993. Handbook of microbiological media. CRC Press, Boca Raton, Fla.
2. **Bakken, L. R., and V. Lindahl.** 1995. Recovery of bacterial cells from soil, p. 9–27. *In* J. T. Trevors and J. D. van Elsas (ed.), Nucleic acids in the environment. Springer Verlag, New York.
3. **Baldwin, E. R.** 1927. Tuberculosis; bacteriology, pathology and laboratory diagnosis, p. 59–61. Lea and Febiger, Philadelphia.
4. **Beloin, R. M., J. L. Sinclair, and W. C. Ghiorse.** 1988. Distribution and activity of microorganisms in subsurface sediments of a pristine study site in Oklahoma. Microb. Ecol. **16:**85–97.
5. **Binder, P., C. Laplace-Builhe, A. Leboeuf, G. Frelat, and R. Masse.** 1988. Analyse de bactéries par cytométrie en flux. Trav. Sci. Chercheurs Serv. Sante Armees **9:**51–52.
6. **Bloem, J., M. Veninga, and J. Shepherd.** 1995. Fully automatic determination of soil bacterium numbers, cell volumes, and frequencies of dividing cells by confocal laser scanning microscopy and image analysis. Appl. Environ. Microbiol. **61:**926–936.
7. **Breed, R. S., and J. D. Brew.** 1925. Counting bacteria by means of the microscope. Technical bulletin 49. N.Y. Agricultural Experiment Station, Geneva, N.Y.
8. **Buck, J. D.** 1979. The plate count in aquatic microbiology. *In* J. W. Costerton, and R. R. Colwell (ed.), Native aquatic bacteria: enumeration, activity and ecology, p. 19–28. American Society for Testing and Materials, Philadelphia.
9. **Christensen, H., L. R. Bakken, and R. A. Olsen.** 1993. Soil bacterial DNA and biovolume profiles measured by flow cytometry. FEMS Microbiol. Ecol. **102:**129–140.
10. **Colwell, R. R.** 1979. Enumeration of specific populations by the most-probable-number (MPN) method, p. 56–61. *In* J. W. Costerton and R. R. Colwell (ed.), Native aquatic bacteria: enumeration, activity and ecology. American Society for Testing and Materials, Philadelphia.
11. **Coulter, W. H.** 1956. High speed automatic blood cell counter and cell size analyzer. Proc. Natl. Electrophoresis Conf. **12:**1034–1042.
12. **Daley, R. J.** 1979. Direct epifluorescence enumeration of native aquatic bacteria: uses, limitations and comparative accuracy, p. 29–45. *In* J. W. Costerton and R. R. Colwell (ed.), Native aquatic bacteria: enumeration, activity and ecology. American Society for Testing and Materials, Philadelphia.
13. **DeLeo, P.** 1996. Factors affecting protozoan predation of bacteria clogging laboratory aquifer microcosms. Ph.D. dissertation. Cornell University, Ithaca, N.Y.

13a. **DeLeo, P., and P. Baveye.** Unpublished data.

14. **Diaper, J. P., and C. Edwards.** 1994. Flow cytometric detection of viable bacteria in compost. FEMS Microbiol. Ecol. **14:**213–220.
15. **Dobbins, D. C., C. M. Aelion, and F. Pfaender.** 1992. Subsurface, terrestrial microbial ecology and biodegradation of organic chemicals: a review. Crit. Rev. Environ. Control **22:**67–136.
16. **Ferry, R. M., L. E. Farr, Jr., and M. G. Hartman.** 1949. The preparation and measurement of the concentration of dilute bacterial aerosols. Chem. Rev. **44:**389–417.
17. **Fry, J. C.** 1988. Determination of biomass. *In* B. Austin (ed.), Methods in aquatic bacteriology, p. 27–72. John Wiley & Sons, New York.
18. **Ghiorse, W. C.** 1993. Use of the confocal laser scanning microscope for detecting viable, but unculturable subsurface bacteria, p. B-33. *In* Proceedings of the Second International Symposium on Subsurface Microbiology, Bath, United Kingdom, 19 to 24 September 1993.

18a. **Ghiorse, W. C.** Personal communication.

19. **Ghiorse, W. C., and D. L. Balkwill.** 1983. Enumeration and morphological characterization of bacteria indigenous to subsurface environments. Dev. Ind. Microbiol. **24:**213–224.
20. **Gucker, F. T., C. T. O'Konski, H. B. Pickard, and J. J. Pitts, Jr.** 1947. A photoelectric counter for colloidal particles. J. Am. Chem. Soc. **69:**2422–2431.
21. **Gucker, F. T., and C. T. O'Konski.** 1949. Electronic methods of counting aerosol particles. Chem. Rev. **44:**373–388.
22. **Haldeman, D. L., P. S. Amy, D. Ringelberg, D. C. White, R. E. Garen, and W. C. Ghiorse.** 1995. Microbial growth and resuscitation alter community structure after perturbation. FEMS Microbiol. Ecol. **17:**27–38.
23. **Halvorson, H. O., and N. R. Ziegler.** 1933. Application of statistics to problems in bacteriology. J. Bacteriol. **25:**101–121.
24. **Haugland, Richard P.** 1992. Handbook of fluorescent probes and research chemicals. Molecular Probes, Inc. Eugene, Oreg.
25. **Herbert, R. A.** 1990. Methods for enumerating microorganisms and determining biomass in natural environments. Methods Microbiol. **22:**1–39.
26. **Hitchens, A. P., and Leikind, M. C.** 1939. The introduction of agar-agar into bacteriology. J. Bacteriol. **37:**485–493.
27. **Hofstraat, J. W., W. J. M. van Zeijl, J. C. H. Peeters, and L. Peperzak.** 1994. Flow cytometry: fast and quantitative characterization of particles in suspension. Anal. Chim. Acta **290:**135–145.
28. **Kackle, V.** 1990. Electrical resistance pulse sizing: Coulter sizing, p. 45–80. *In* M. R. Melamed, T. Lindmo, and M. L. Mendelsohn (ed.), Flow cytometry and sorting, 2nd ed. Wiley-Liss, N.Y.
29. **Lebaron, P., and F. Joux.** 1994. Flow cytometric analysis of the cellular DNA content of *Salmonella typhimurium* and *Alteromonas haloplanktis* during starvation and recovery in seawater. Appl. Environ. Microbiol. **60:**4345–4350.
30. **Lebaron, P., M. Troussellier, and P. Got.** 1994. Accuracy of epifluorescence microscopy counts for direct estimates of bacterial numbers. J. Microbiol. Methods **10:**89–94.
31. **Lee, M. D., J. M. Thomas, R. C. Borden, P. B. Bedient, C. H. Ward, and J. T. Wilson.** 1988. Biorestoration of aquifers contaminated with organic compounds. Crit. Rev. Environ. Control **18:**29–89.
32. **Lindahl, V., and L. R. Bakken.** 1995. Evaluation of methods for extraction of bacteria from soil. FEMS Microbiol. Ecol. **16:**135–142.
33. **Lloyd, D.** 1993. Flow cytometry: a technique waiting for microbiologists, p. 1–10. *In* D. Lloyd (ed.), Flow cytometry in microbiology. Springer-Verlag, London.
34. **Melamed, M. R., P. F. Mullaney, and H. M. Shapiro.** 1990. An historical review of the developments of flow cytometers and sorters, p. 11–25. *In* M. R. Melamed, T. Lindmo, and M. L. Mendelsohn (ed.) Flow cytometry and sorting, 2nd ed. Wiley-Liss, New York.
35. **Moldavan, A.** 1934. Photo-electric technique for the counting of microscopical cells. Science **80:**188–189.
36. **Page, S., and R. G. Burns.** 1991. Flow cytometry as a means of enumerating bacteria introduced into soil. Soil Biol. Biochem. **23:**1025–1028.
37. **Ploem, J. S., and H. J. Tanke.** 1987. Introduction to fluorescence microscopy, p. 27–33. Oxford University Press, London.
38. **Porter, J., C. Edwards, J. A. W. Morgan, and R. W. Pickup.** 1993. Rapid, automated separation of specific bacteria from lake water and sewage by flow cytometry and cell sorting. Appl. Environ. Microbiol. **59:**3327–3333.
39. **Shapiro, H.** 1995. Practical flow cytometry, 3rd ed., p. 43–74. Wiley-Liss, New York.
40. **Simon, N., N. LeBot, D. Marie, F. Partensky, and D. Vaulot.** 1995. Fluorescent in-situ hybridization with rRNA-targeted oligonucleotide probes to identify small phytoplankton by flow cytometry. Appl. Environ. Microbiol. **61:**2506–2513.
41. **Sinclair, J. L., and W. C. Ghiorse.** 1989. Distribution of aerobic bacteria, protozoa, algae and fungi in deep subsurface sediments. Geomicrobiol. J. **7:**15–31.
42. **Slepecky, R. A., and H. E. Hemphill.** 1992. The genus *Bacillus*-nonmedical. *In* A. Balows (ed.), The prokaryotes: a handbook on the biology of bacteria, p. 1663–1696. Springer-Verlag, New York.
43. **Stanley, P. M., M. A. Gage, and E. L. Schmidt.** 1979. Enumeration of specific populations by immunofluorescence, p. 46–55. *In* J. W. Costerton and R. R. Colwell (ed.), Native aquatic bacteria: enumeration, activity and ecology. American Society for Testing and Materials, Philadelphia.
44. **Steen, H. B.** 1990. Flow cytometric studies of microorganisms, p. 605–622. *In* M. R. Melamed, T. Lindmo, and M. L. Mendelsohn (ed.), Flow cytometry

and sorting, 2nd ed. Wiley-Liss, New York.
45. **Steen, H. B., K. Skarstad, and E. Boye.** 1990. DNA measurements of bacteria. Methods Cell Biol. **33:**519–526.
46. **Vandevivere, P., and P. Baveye.** 1992. Saturated hydraulic conductivity reduction caused by aerobic bacteria in sand columns. Soil Sci. Soc. Am. J. **56:**1–13.
47. **Vesey, G., P. Hutton, A. Champion, N. Ashbolt, L. K. Williams, A. Warton, and D. Veal.** 1994. Application of flow cytometric methods for the routine detection of *Cryptosporidium* and *Giardia* in water. Cytometry **16:**1–6.
48. **Vesey, G., J. S. Slade, M. Byrne, K. Shepherd, P. J. Dennis, and C. R. Fricker.** 1993. Routine monitoring of *Cryptosporidium* oocysts in water using flow cytometry. J. Appl. Bacteriol. **75:**87–90.
49. **Vesey, G., J. S. Slade, M. Byrne, K. Shepherd, and C. R. Fricker.** 1993. A new method for the concentration of *Cryptosporidium* oocysts from water. J. Appl. Bacteriol. **75:**82–86.
50. **Volsch, A., W. F. Nader, H. K. Geiss, G. Nebe, and C. Birr.** 1990. Detection and analysis of two serotypes of ammonia-oxidizing bacteria in sewage plants by flow cytometry. Appl. Environ. Microbiol. **56:**2430–2435.
51. **Wallner, G., R. Erhart, and R. Amann.** 1995. Flow cytometric analysis of activated sludge with rRNA-targeted probes. Appl. Environ. Microbiol. **61:** 1859–1866.
52. **Wilson, J. T., J. F. McNabb, D. L. Balkwill, and W. C. Ghiorse.** 1983. Enumeration and characterization of bacteria indigenous to a shallow water-table aquifer. Ground Water **21:**134–142.
53. **Yagoda-Shagam, J., L. L. Barton, W. P. Reed, and R. Chiovetti.** 1988. Fluorescein isothiocyanate-labeled lectin analysis of the surface of the nitrogen-fixing bacterium *Azospirillum brasilense* by flow cytometry. Appl. Environ. Microbiol. **54:**1831–1837.